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ORIGINAL ARTICLE

Modification of esterified cell wall phenolics increases vulnerability of tall fescue to herbivory by the fall armyworm

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Abstract Feruloylation of arabinoxylan in grass cell walls leads to cross-linked xylans. Such cross-linking appears to play a role in plant resistance to pathogens and insect herbivores. In this study, we investigated the effect of ferulate cross-linking on resistance to herbivory by fall armyworm (*Spodoptera frugiperda*) making use of genetically modified tall fescue [*Schedonorus arundinaceus* (*Festuca arundinacea*)] expressing a ferulic acid esterase gene. Mature leaves of these plants have significant reduced levels of cell wall ferulates and diferulates but no change in acid detergent lignin. These reduced levels of esterified cell wall ferulates in transgenic plants had a positive effect on all measures of armyworm larval performance examined. More larvae survived (89 vs. 57 %) and grew faster (pupated 2.1 days sooner) when fed transgenic leaves with reduced levels of cell wall ferulates, than when fed control tall fescue leaves where levels of cell wall ferulates were not altered. Overall, mortality, growth and food utilization were negatively associated with level of esterified cell wall ferulates and diferulates in leaves they were fed. This study is the first to use transgenic plants with modified level of cell wall esterified ferulates to test the role of feruloylation in plant resistance to insects. It is concluded that the accumulation of ferulates and the cross-linking of arabinoxylans via diferulate esters in the leaves of tall fescue underlies the physical barrier to insect herbivory. Reducing ferulate cross-linking in grass cell walls could increase susceptibility of these plants to insect folivores.

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Abbreviations

AX	Arabinoxylan
GAX	Glucuronoarabinoxylan
FA	Ferulic acid
HCA	Hydroxycinnamic acid
FAW	Fall armyworm
FAEA	Ferulic acid esterase
AD	Approximate digestibility
ECI	Efficiency of conversion of ingested food
ECD	Efficiency of conversion of digested food into biomass
RGR	Relative growth rate
GLM	General linear model
SE	Standard error
tFA	*Trans*-ferulic acid
cFA	*cis*-ferulic acid
tpCA	*Trans p*-coumaric acid
5-5′ DFA	5-5′-diferulic acid
8-0-4′ DFA	8-0-4′-diferulic acid
8-5C DFA	8-5cyc diferulic acid benzofuran

Introduction

Plants rely on their cell walls to provide shape and strength to cells, to glue cells together, to give rigidity to the whole plant, and to function as a physical barrier that impedes pathogen attack (Brett and Waldron 1996). The growing cell wall consists of cellulose microfibrils embedded in a matrix of hemicelluloses, pectins, proteins and phenolic substances (Carpita and Gibeaut 1993; Carpita and Mccann 2000). Grass cell walls are distinctive in composition, with

xylans, to a significant extent, usurping the roles of pectin and xyloglucan in the primary walls of dicotyledonous plants (Carpita 1996; Carpita and Gibeaut 1993). The xylan of primary walls typically is a complex molecule with a backbone of 1,4-linked β-D-xylopyranose residues substituted with arabinose and glucuronic acid side chains, and is called arabinoxylan (AX) or glucuronoarabinoxylans (GAX) (Whilstler and Richards 1970). A proportion of the arabinose side chains are further substituted with acidic residues, notably ferulic (4-hydroxy-3-methoxycinnamic), *p*-coumaric (4-hydroxycinnamic) and 4-0-methylglucuronic acids (Hartley 1972).

Ferulic acid (FA) is the major hydroxycinnamic acid (HCA) identified in both primary and secondary grass cell walls, being abundant in the epidermis, xylem vessels, bundle sheets and sclerenchyma (Faulds and Williamson 1999). Levels of FA in cell walls can be as high as 3 % w/w (Wende and Fry 1997). During cell wall deposition and lignification, peroxidase-mediated oxidative coupling of FA residues results in the formation of dehydrodimers, trimers and tetramers, which function to cross-link xylans (Bunzel et al. 2006; Funk et al. 2005; Ralph et al. 1994b). The bi-functional nature of FA also allows feruloyl polysaccharide esters to participate with lignin monomers in oxidative coupling pathways to generate a ferulate–polysaccharide–lignin complex through ester–ether linkages (Jacquet et al. 1995).

Leaf structural traits are thought to pose a predicament especially for herbivores to overcome as they eat and digest leaf tissue. Resistance to fracture propagation, or leaf toughness, is considered to be a critical biomechanical property for herbivorous chewing insects (Clissold et al. 2006, 2009; Sanson 2006). Factors affecting leaf toughness include the amount and arrangement of cell wall constituents (Hunt et al. 2008; Lucas et al. 2000; Read and Stokes 2006). In addition, cell walls are reservoirs of secondary metabolites that inhibit feeding of insects (Heldt 2011). Grasses normally infected with fungal endophytes generally lack plant secondary metabolites when endophytes are absent (Kuldau and Bacon 2008; Osbourn 2003; Tscharntke and Greiler 1995). Therefore, grass leaf biomechanical properties are likely to be an important means of defense against herbivory.

Accumulation of cell wall ferulates and the cross-linking of xylan appear to play a role in different plant processes such as in cell wall growth and extensibility, as well as in cell wall disassembly during ruminant digestion and industrial saccharification for biofuel production. There is also some evidence in the literature that FA cross-linking is involved in plant protection against insects and pathogens (Barros-Rios et al. 2011; Bergvinson et al. 1997; Bily et al. 2003; Santiagio et al. 2006), where the ferulate-polysaccharide–lignin complex is likely to contribute to strengthening the cell wall. However, the specific role of AX feruloylation in the processes of plant resistance to herbivorous pests and diseases has been established largely by indirect experiments.

Tall fescue [*Schedonorus arundinaceus* (Schreb.), Dumort = *Lolium arundinaceum* (Schreb.) Darbysh., formerly known as *Festuca arundinacea* Schreb. (Barkworth et al. 2007)] is important in forage/livestock systems, forming the plant basis for beef and milk production worldwide. This grass is also a potential feedstock crop for biofuel production, being perennial, resistant to adverse growing conditions, suitable for low quality land, and with high water soluble carbohydrate content. It is widely planted in the USA where it grows on an estimated 35 million acres (Sleper and West 1996). Several insect herbivores (e.g., scarab beetle grubs, billbugs, aphids, mealybugs, leafhoppers and caterpillars) feed on different parts (e.g., leaves, seeds, sap, and roots) of tall fescue reducing its productivity and persistence in both agricultural and turf situations. One of these insects is the leaf grazing fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae). In the USA, this herbivore is one of the most likely to attack tall fescue in both pastures and turf with damage to tall fescue often severe in the Gulf Coast states, especially in late summer and fall (Bergvinson et al. 1997; Braman et al. 2002). The interaction between FAW and tall fescue has been well studied (Clay and Schardl 2002) compared to the other insect pests of tall fescue, suggesting it is the best insect model for examining the role of AX feruloylation in resistance to insect herbivores.

We previously generated transgenic *Lolium perenne* (Buanafina et al. 2006) and tall fescue (Buanafina et al. 2008) plants with significantly reduced levels of cell wall esterified ferulates and diferulates by over-expression of a vacuole-targeted *Aspergillus niger* ferulic acid esterase gene (*faeA*) released on cell death, as well as tall fescue plants constitutively expressing FAEA targeted to the apoplast and golgi (Buanafina et al. 2010). These plants have increased levels of FAEA expression, and reduced levels of ferulates esterified into the growing cell wall but no change in either neutral or acid detergent fiber or in acid detergent lignin in mature leaves (Buanafina et al. 2010).

Here we used some of these transgenic plants to test the hypothesis that reduction of cell wall ferulates and cross-linking of AX by diferulate esters in leaves of grasses effect herbivore performance. We report here the performance of FAW larvae feeding on transgenic and control tall fescue leaves in terms of the standard criteria (survival, growth and food assimilation) for investigating food quality of caterpillar herbivores that reflect the potential for field population growth. We also examined co-variation between FAW performance and the levels of HCAs in transgenic and control leaves fed to this herbivore.

Materials and methods

Plant material

Four lines of tall fescue were included in this study: a control (C) (non-transgenic) and three transgenic lines expressing an *A. niger* ferulic acid esterase. Two of the transgenic lines (T10 and T11) had ferulic acid esterase (FAEA) targeted to the apoplast and one transgenic line (T21) had FAEA targeted to the Golgi. Transgenic plants were produced by particle bombardment of an embryogenic suspension culture of tall fescue genotype (20BN3) from cultivar S170 with two gene constructs carrying a genomic clone of *faeA* from *A. niger*. The vectors in question were made in the pCOR105 plasmid under the rice actin promoter with the potato protease inhibitor II (PPI) apoplast motif added to confer apoplast targeted FAEA expression (T10 and T11) or a rat sialyltransferase motif for Golgi targeting (T21) as described previously (Buanafina et al. 2008, 2010). The background genotype used was clonal plants of 20BN3, which was also used as the control in all experiments reported. Characterization of the transgenic plants used in this study, such as gene integration, FAEA activity, effect of different intracellular targeted FAEA expression (apoplast, Golgi, ER) on the levels of esterified cell wall ferulates, sugar composition, and lignification, compared to control, is reported in Buanafina et al. (2010).

Plant growth conditions

Vegetatively propagated tillers were used to produce numerous clones of transgenic and control tall fescue plants. Tillers were planted into 6 inch diameter pots containing a 5:1 mixture of Miracle-Gro Potting Mix (The Scotts Company, Marysville, OH, USA) and vermiculate. All plants were grown in a controlled environment chamber at 22/16 °C (day/night) temperature, 16 h photoperiod, 180 µmol photos m^{-2} s^{-1} photosynthetically active radiation and 40 % humidity. Eight to ten pots were grown per line each randomly arranged in the growth chamber for the duration of the experiment and fertilized at 4-week intervals with a solution containing 1.1 g l^{-1} N, 0.28 g l^{-1} P and 1.0 g l^{-1} K. *Neoseiulus cucumeris* (Oudemans) and *Stratiolaelaps scimitus* (Womersley) (IPM Laboratories, Locke, NY, USA) were used as biological control for thrips, aphids and fungus gnats.

Leaf samples for insect feeding, cell wall HCAs and enzyme digestion analysis

The basal segment (10 cm) of the newest fully extended, 3rd leaf blade within a tiller was used for insect feeding experiments and chemical analyses. At the time of sampling, each pot contained at least 25 tillers. A tiller was never resampled for leaf material and we waited 3 days before obtaining leaves from plants containing tillers that had been previously sampled. As it was not logistically possible to test all three transgenic genotypes at the same time, each genotype was tested in separate experiments, each including control plants.

Analyses of cell wall hydroxycinnamic acids in leaf blades

Two to three leaf blade basal segments from transgenic and control lines were harvested on different days during each larval feeding experiment. Quantitative analysis of ester-bound HCAs, carried out on freeze-dried powdered leaf material, was performed as previously described in Buanafina et al. (2008) with minor modifications. Briefly, soluble phenolics and chlorophyll pigments were extracted with methanol. Insoluble pellets were dried, weighed and their ester-bound compounds extracted with 1 M NaOH, and recovered following acidification, on an activated reverse phase C_{18} µSep-Pak column (Waters Inc.) eluted with 100 % methanol. Extracts were analyzed by HPLC on a Nova-Pak C18 4 µm (3.9 × 75 mm) column (Waters, Inc.) in 100 % methanol–5 % acetic acid, with a 10–80 % methanol gradient over 30 min at a flow rate of 1 ml/min. Phenolic compounds were detected and quantified with a Waters 996 photo-diode array detector as in Buanafina et al. (2010).

Determination of FAEA activity

FAEA activity was determined in soluble extracts of fresh leaves (0.5 g of newest fully extended, 3rd leaf blades within a tiller) as in Buanafina et al. (2008) to confirm expression of the gene in transgenic lines. One unit of FAEA specific activity equals 1 µg of ferulic acid released in 24 h at 28 °C per gram of fresh weight.

Cellulase-mediated sugar release from grass leaves

Freeze-dried, powdered basal leaf material (10 mg), prepared as for cell wall analysis, was re-hydrated in extraction buffer (0.1 M sodium acetate, pH 5.5). After centrifugation, the supernatant was removed and 40 µl (Exp 1), 80 µl (Exp 2) and 150 µl (Exp 3) of cellulase (*T. reseei*, Sigma, 63 units/ml) was added to each sample. Volume was adjusted to 1 ml with extraction buffer and incubated at 35 °C for 24 h. Procedures for reducing sugar determination by the ρ-hydroxybenzoic acid hydrazide method were as in Buanafina et al. (2010).

Source of insects

Eggs of the rice plant host strain of FAW were obtained from a lab-reared colony of R. Meagher's at the Insect Behavior and Biocontrol Research Unit in the USDA ARS Center for Medical, Agricultural and Veterinary Entomology (Gainesville, FL). This colony was reared (Guy et al. 1985) in mass culture for 53, 55 and 59 generations, with respect to experiment, on a pinto bean-based artificial diet. It originated from >200 larvae collected from pasture grasses at the Range Cattle Research and Education Centre, Ona, Hardee Co., FL, USA between May 2003 and October 2003, and was named OnaR. Individuals used to establish this colony had the RFLP marker that is associated with the rice strain (Nagoshi et al. 2006). Purity of this colony is confirmed by periodic genotyping of randomly chosen individuals by R. Meagher and R. Nagoshi. A subsample of insects ($N = 10$ per experiment) was genotyped to confirm that the rice plant host strain was used in our experiments.

Larval growth performance

Newly hatched neonate larvae were placed on a particular diet treatment held in a Petri dish (100 × 15 cm) covered on the inside bottom with filter paper. Diet treatments consisted of artificial diet (Bio-Serv, Frenchtown, NJ, USA), control tall fescue leaves, or leaves from transgenic lines (T10, T11, or T21 as described above). Number of neonates used at the start of an experiment varied with diet treatment (25 for artificial diet, 50 for control leaves, and 30 for transgenic leaves) so that about 25 larvae would reach pupation. Larvae were allowed to feed ad libitum throughout the experiment with fresh leaves added every other day until larvae on control leaves reached the third instar after which fresh leaves of all lines being tested were added every day. Dishes containing larvae and food were held in an environmental chamber at 27 °C, 70 % relative humidity (RH), and 14:10 L:D photoperiod. Beginning on the sixth day after hatching, larvae were weighed daily to the nearest milligram through pupation. Mortality at specific times during development, days to pupation and relative growth rate (RGR) based on fresh weights at 8 and 10 days after hatching were also used as measures of insect performance.

Several arithmetic and geometric approaches to estimating RGR over a 2-day period (8–10 days after hatching) were explored including that reported by Massey et al. (2006), Farrar et al. (1989), Barbehenn et al. (2004), and an approach widely used for plant growth and often for insect growth (Radford 1967; Hoffmann and Poorter 2002; RGR = [ln(weight at day 10) − ln(weight at day 8)]/2). The plant growth approach was highly linearly correlated ($P < 0.0001$, $r^2 \geq 0.96$) with all other approaches, whether or not they were square root or arcsine square root transformed. Although each of these approaches did not alter the patterns observed or ensuing conclusions, the plant growth approach was used in RGR calculations presented herein because it was the only approach that yielded a good normal distribution without requiring further transformation.

Larval food utilization experiment

Newly hatched neonate larvae were placed in clear plastic 30 ml cups containing artificial Lepidoptera diet (Bio-Serv, Frenchtown, NJ, USA). All components of this experiment were performed in the environmental chamber. A large preliminary experiment to discern criteria for selection of developmentally synchronous last instars was performed as described in Fescemyer et al. (1986). This experiment determined that pentultimate, fourth instars with slipped head capsules and weighing greater than 160 mg 10–12 h after lights on (ALO) pupated during the fifth day of the ultimate, fifth instar. Pentultimate larvae fitting these developmental criteria were individually placed without food in a Petri dish (100 × 15 mm). These larvae subsequently molted during the scotophase. An experiment using 10 larvae per food treatment was begun 2–3 h ALO in the following photophase (i.e., first day of the ultimate instar) by weighing each larva, which was then provided with a known weight of food from a particular diet treatment. Food treatments (about 500 mg in wet weight) consisted of artificial diet, non-transgenic control tall fescue leaves, or leaves from a transgenic line (T10, T11, or T21; described above). Larvae were allowed to feed for 24 h during which all food provided was consumed. Each larva was then transferred to its own, fresh Petri dish where they were starved for another 24 h, to allow all frass to pass, before being reweighed. The dry weights of each larva and frass collected from each larva were measured separately after lyophilization. A preliminary experiment determined that the amount of food provided would be consumed within 24 h with dissection and observation of larval guts used to determine that all frass passed in the following 24 h of starvation. Values of water content for larvae and food, derived from samples of the same larval or food sources, were used to convert fresh masses of larvae and food to dry mass. The direct and converted dry determinations of larval mass, food intake and frass were used to calculate food utilization efficiency measures according to Raps and Vidal (1998).

Statistical analysis

All statistical analyses were performed with JMP™, version 5 (SAS Institute Inc., Cary, NC, USA). Values given in the text are means plus or minus the standard error (SE). Probability (P) values in the results section are for the independent effects type III sums of squares F test from a

standard least squares general linear model (GLM). It was not logistically possible to test all three transgenic lines at the same time. Therefore, each line was tested in separate experiments, each including the control and artificial diet treatments. The model for HCAs considering the fixed factors experiment (block; $N = 3$) and genotype ($N = 4$) and their interaction as sources of variation. For larval performance, sources of variation were the fixed factors experiment (block; $N = 3$), diet ($N = 4$) and their interaction. Tukey's test was used to determine significant differences ($\alpha = 0.05$) between the means of main effects. Linear correlations among HCAs were calculated using the Pearson product-moment correlation coefficient. Proportional and relative values were transformed using the arcsine square root unless otherwise indicated. It was not possible to measure HCAs in every leaf fed to larvae. Therefore, levels of HCAs were measured in replicate subsamples of leaves fed to larvae over the course of the experiment. The mean of these HCA measurements was used in statistical analyses to determine if insect performance, except for larval mortality, and food utilization parameters varied with level of HCA in plant cell walls. Each insect was a replicate in all insect parameters measured except for mortality, which was a proportion of all neonates placed on a particular plant treatment within an experiment. Therefore, degrees of freedom were increased when examining how mortality varied with level of HCA in plant cell walls using the mean of this mortality proportion compared to levels of HCAs in replicate leaf subsamples for a particular plant treatment within an experiment.

Results

Characterization of transgenic and non-transformed control plants

We previously looked carefully at the stability of FAEA expressing plants and found that FAEA expression was stable in plants vegetatively propagated via tillering (see supplement data in Buanafina et al. 2008) and by meristem culture, over different generations. Extracted FAEA activity was stable for at least 24 h at 28 °C. Further characterization of transgenic plants used for this study, such as gene targeting, integration of fungal *faeA* into the genome, and the total plant levels of cell wall HCAs compared to the control are reported in Buanafina et al. (2010).

FAEA activity of leaf extracts

FAEA activity in extracts of basal leaf blades following expression of the *faeA* gene was confirmed in all transgenic lines tested. The T11 line had the highest (725 units) activity followed by T10 (648 units) and T21 (618 units). No activity was detected in control plants.

Cell wall hydroxycinnamic acids

Levels of individual and total ferulate monomers [*trans*-ferulic acid (tFA) and *cis*-ferulic acid (cFA)], individual and total ferulate dimers (5-5′-diferulic acid, 5-5′ DFA; 8-0-4′-diferulic acid, 8-0-4′ DFA; and 8-5cyc, 8-5C DFA: diferulic acid benzofuran form), and esterified *trans p*-coumaric acid (tpCA) were lower in the basal leaf blades from transgenic compared to control plants ($P < 0.0001$) (Fig. 1a, b). Differences between experiments were observed for tFA ($P = 0.0079$), cFA ($P = 0.0002$), total ferulate monomers ($P = 0.0011$), 8-0-4′ DFA ($P < 0.0001$) and total ferulate dimers ($P = 0.0006$), but not for tpCA ($P = 0.0823$), 5-5′ DFA ($P = 0.0838$) and 8-5C DFA ($P = 0.5383$). There were no interactions ($P \geq 0.11$) between experiment (i.e., block) and treatment (i.e., plant genotype). Overall, the model explained 74 ± 5 % of the variation in the HCA variables tested.

Across all plant genotypes tested and experiments, there were positive linear correlations ($N = 41$) among all HCA variables tested. Highest correlation coefficients (>0.9) were for tFA with total ferulate monomers, and 8-0-4′ DFA with total diferulate dimers, which is not surprising because tFA and 8-0-4′ DFA comprise 84.8 ± 0.2 and 62.6 ± 0.7 % of total ferulate monomers and total ferulate dimers, respectively. Correlation coefficients of 0.8–0.9 were for total ferulate monomers with total ferulate dimers, tFA with 5-5′ DFA and 8-5C DFA, cFA with total ferulate monomers and total ferulate dimers, total ferulate monomers with 5-5′ DFA and 8-5C DFA with cFA, total ferulate dimers with 5-5′ DFA and 8-5C DFA. Linear correlations among all other HCA variables had coefficients of 0.5–0.7 with most between 0.7 and 0.8.

Cellulase-mediated sugar release from basal leaf blades

To confirm that expression of FAEA, leading to reduction of ferulates and diferulates esterified to AX, also enhances release of cell wall sugars, we digested transgenic and control basal leaf blades with and without *T. reseei* cellulase and measured the amount of reduced sugars released. There was an increase ($P < 0.0001$) in the release of reduced sugars from all transgenic lines in the presence of cellulase compared to control plants (data not shown).

Larval survival and growth performance

Most mortality occurred during the first 5 days of larval development (i.e., early larval mortality) (Fig. 2). Percentage early larval mortality was most highly linearly correlated

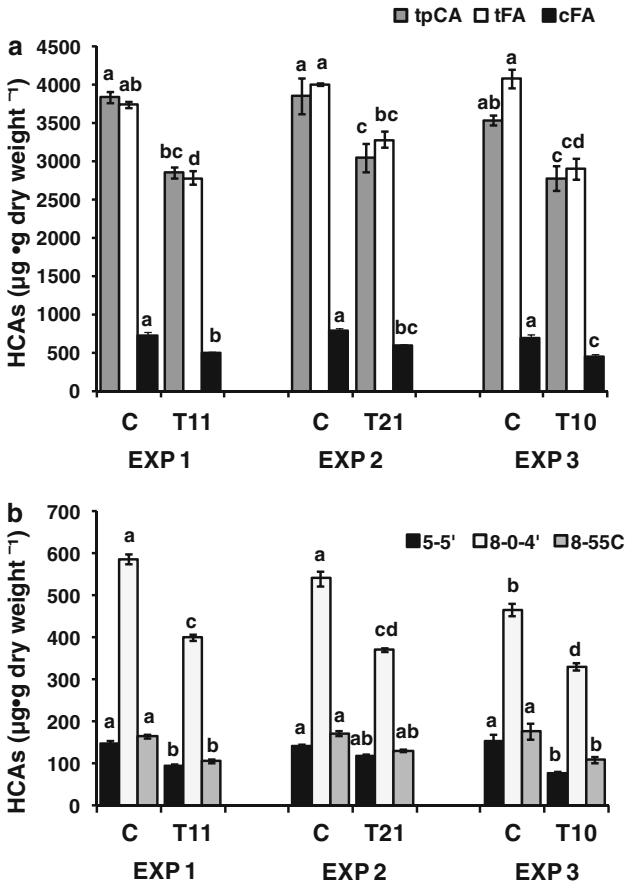

Fig. 1 Levels of ester-bound **a** coumarate (*tpCA trans p*-coumaric acid) and ferulate monomers(*tFA trans*-ferulic acid, *cFA cis*-ferulic acid) and **b** ferulate dimers (5-5′: diferulic acid; 8-0-4′:diferulic acid, and 8-5cyc: diferulic acid benzofuran form), in basal leaf blades of three different tall fescue transgenic lines [T11, T21 or T10 used in experiment (EXP) 1–3, respectively] expressing ferulic acid esterase (FAEA) compared with a non-transformed control line (C). These were the lines on which insect performance was tested. *Bars* are mean ± SE ($N = 4$–10). *Letters at the top of bars* indicate significant difference (Tukey's, $\alpha = 0.05$) within an HCA across experiments

($P < 0.0001$, $r^2 = 0.91$, $y = 0.75x - 5.34$) with percentage mortality occurring from the beginning of larval development through adult eclosion (i.e., total mortality). Therefore, tests for effect of treatments are only reported for these mortality values. Percentage mortality during early larval development was greatest ($P = 0.005$) (Fig. 2) when larvae ate control plants followed by transgenic plants and artificial diet. Differences in mortality were not observed ($P = 0.6$) between experiments and there were not enough degrees of freedom to test the interaction between experiment and diet treatment. A similar trend was observed for percentage total mortality ($P = 0.01$ for diet treatment; $P = 0.3$ for experiment) (Fig. 2). Overall, this main effects model explained 92 and 90 % of the variation in early larval and total mortality observed, respectively.

Duration of the larval stage was affected by larval diet treatment ($P < 0.0001$) (Fig. 3). In particular, larvae that ate

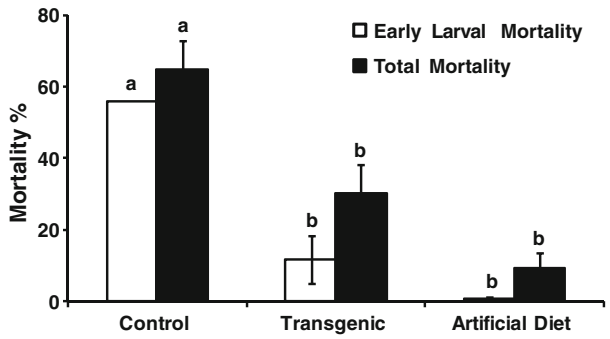

Fig. 2 Percentage mortality of FAW larvae fed leaf bases of control tall fescue compared to those that fed transgenic plants or artificial diet. *Bars* are mean ± SE ($N = 3$). *Bars with different letters* are significantly different (Tukey's, $\alpha = 0.05$) within a mortality measure

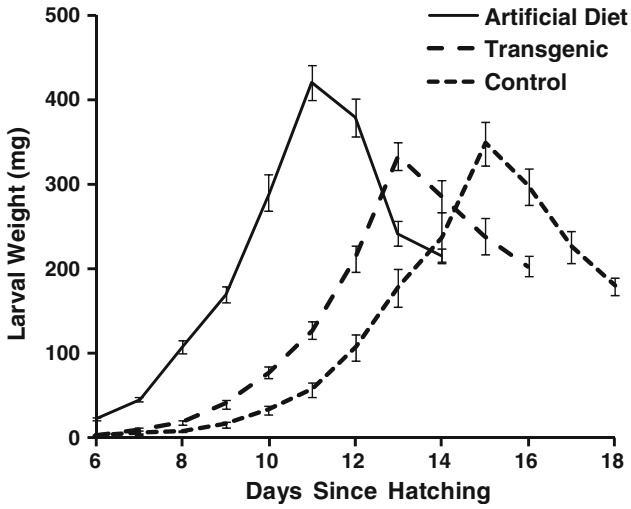

Fig. 3 Weight and growth of FAW larvae fed artificial diet or leaf bases from tall fescue transgenic or non-transformed control plants. *Bars* are mean ± SE ($N = 41$)

transgenic plants had a shorter (16.2 ± 0.2 days) larval stage by 2.1 days than those that ate control plants (18.3 ± 0.2 days). Larvae that ate artificial diet had the shortest (14.4 ± 0.2 days) larval stage, pupating 1.8 and 3.9 days faster than larvae that ate transgenic and control plants, respectively. Pupal weight was also affected by larval diet treatment ($P < 0.0001$) (Fig. 3) with larvae that ate artificial diet developing into the heaviest pupae (215 ± 3 mg). Larvae that ate transgenic plants developed into pupae that were not different in weight (164 ± 2 mg) from those that ate control plants (177 ± 4 mg), suggesting that larvae eating control plants were compensating by spending a longer period of time in the larval stage. Differences ($P \geq 0.21$) were not observed between experiments, genders, and for interactions among main effects. Overall, the main effects model explained 61 and 52 % of the variation in duration of the larval stage and pupal weight, respectively.

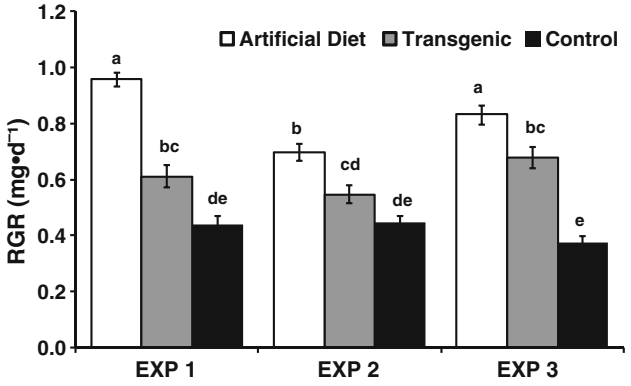

Fig. 4 Relative growth rate (RGR) of FAW larvae fed artificial diet or leaf bases of tall fescue transgenic lines [T11, T21 or T10 used in experiment (EXP) 1–3, respectively] or non-transformed control plants. *Bars* are mean ± SE ($N = 13$–30). *Bars with different letters* are significantly different (Tukey's, $\alpha = 0.05$) across a diet

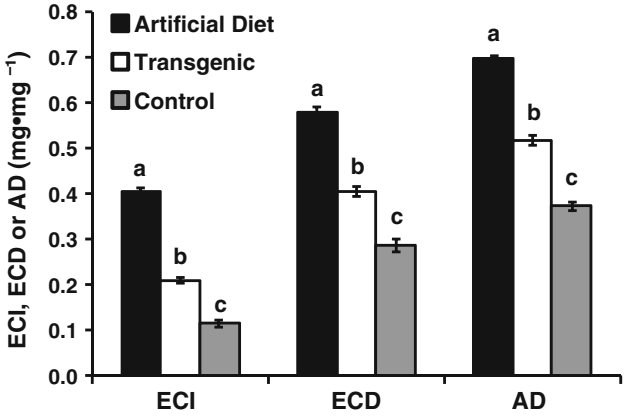

Fig. 5 Effect of eating artificial diet or leaf bases of tall fescue transgenic or non-transformed control plants on the ability of FAW larvae to assimilate their food. Assimilation is reported as efficiency of conversion of ingested food (ECI), approximate digestibility (AD) and efficiency of conversion of digested food into biomass (ECD). *Bars* are mean ± SE ($N = 10$). *Bars with different letters* are significantly different (Tukey's, $\alpha = 0.05$) within a measure of assimilation

Diet eaten by larvae affected RGR ($P < 0.0001$) with larvae that ate artificial diet having the highest RGR followed decreasingly by larvae that ate transgenic leaves and control leaves (Fig. 4). The RGR also differed by experiment ($P = 0.0003$) and interaction between experiment and diet treatment ($P = 0.0001$) (Fig. 4). Within an experiment, larvae that ate artificial diet grew faster than those that ate transgenic leaves who in turn grew faster than those that ate control leaves.

Food assimilation

Approximate digestibility (AD) was linearly correlated ($P < 0.0001$, $r^2 = 0.82$, $y = 0.75x - 0.16$) to efficiency of conversion of ingested food (ECI) and to efficiency of conversion of digested food into biomass (ECD), but less variation was explained by the later ($P < 0.0001$, $r^2 = 0.51$, $y = 0.67x + 0.07$). There also was a linear correlation between ECI and ECD ($P < 0.0001$, $r^2 = 0.84$, $y = 0.83x - 0.11$). Therefore, subsequent analyses are only reported for AD and ECI.

Diet eaten by larvae differed in AD ($P < 0.0001$) with artificial diet having the highest AD followed decreasingly by larvae that ate transgenic leaves and control leaves (Fig. 5). There was no difference in AD with experiment ($P = 0.5$) or interaction between experiment and diet treatment ($P = 0.12$). Larvae were better ($P < 0.0001$) at converting (ECI) artificial diet into body mass than when eating transgenic leaves that were in turn converted better than control leaves (Fig. 5). The ECI did not differ with experiment ($P = 0.17$), but there was an interaction between experiment and diet treatment ($P = 0.001$).

Variation in insect performance with level of HCAs in plant cell walls

Stepwise regression was used in a backward elimination approach with each dependent insect parameter variable to test which of the independent HCA variables measured can be deleted without appreciably increasing the residual sum of squares. This approach was used because level of each HCA measured in plant leaves fed to larvae correlate well with each other in a positive direction as indicated above. Therefore, it was not surprising that level of total ferulate monomers and total ferulate dimers could be deleted from the standard least squares GLM testing the effect of hydroxycinnamic acid level in plant cell walls on insect performance. Level of tpCA could also be deleted from this model, whose independent HCA variables now consisted of tFA, cFA, 5-5′ DFA, 8-0-4′ DFA and 8-5C DFA.

Early larval mortality, all measures of growth performance (pupal weight, larval duration, RGR), and all measures for efficiency of food utilization (ECI, ECD, or AD) were affected by each of the individual ferulate monomers and dimmers measured. The high degree of linear correlation among these HCA variables eliminated determination of which of these HCAs explained more strongly the variation on mortality. Mortality ($P < 0.0001$) and larval duration ($P < 0.0001$) increased with increasing level of the individual FA monomers and dimmers measured. Pupal weight ($P = 0.007$), RGR ($P < 0.0001$), ECI ($P < 0.0001$), ECD ($P < 0.0001$), and AD ($P < 0.0001$) decreased comparable to increasing level of the individual FA monomers and dimmers measured. There were not enough degrees of

freedom (i.e., 5, 54) in the models to test interactions among the HCAs measured.

Discussion

As discussed in Buanafina et al. (2010), apoplast and Golgi targeted FAEA expression in tall fescue plants resulted in the release of not only esterified ferulic acid but also ferulate dimers and *p*-coumarate from the cell wall of these plants. The present study used some of these transgenic plants to test the significance of cell wall ferulates, diferulates and *p*-coumarates in protecting plants against herbivorous insects. Esterification of AX by ferulates facilitates the cross-linking of AX through the formation of diferulates, largely determining the degree of xylan cross-linking in cell walls of tall fescue. We showed that significantly reduced levels of these cell wall ferulates affected all measures of FAW larval performance examined. More larvae survived and grew faster when fed FAEA transgenic, than when fed control tall fescue leaves. These larvae utilized more of the transgenic leaves they were fed than those fed control leaves. Overall, mortality, growth and food utilization were negatively associated with level of esterified cell wall HCA in leaves they were fed.

Indirect experimentation has been primarily used to establish the specific role that feruloylation plays in protecting plants from herbivorous insects (Bergvinson et al. 1997; Santiagio et al. 2006; Barros-Rios et al. 2011). Although previous research examined possible effects of non-modified bioenergy grasses on insect performance (Dowd and Johnson 2009; Nabity et al. 2011), our study is the first to use transgenic plants with modified level of cell wall esterified ferulates to demonstrate that reduction of these cell wall HCAs resulted in plants more susceptible to insect folivores which imply a mechanical restriction to feeding or assimilation. Herbivory is one of the most widespread sources of biotic damage to tall fescue and other plants. Plant cell wall components are important factors dictating plant resistant to mechanical injury and the tearing action of mandibles (Read and Stokes 2006; Clissold 2008). Force to fracture leaves increases with the proportion of sclerenchyma in the cross-sectional area of the leaf, energy to fracture increases with the number of fibrous vascular bundles (Vincent 1991; Wright and Illius 1995), and leaves more sclerophyllous tend to be stronger and tougher (Turner 1994; Read and Stokes 2006). Ferulic acid is the major HCA in grasses and particularly abundant in the xylem vessels, bundle sheets and sclerenchyma (Faulds and Williamson 1999; Harris and Hartley 1976; Hartley and Ford 1989). It is likely that decreased xylan cross-linking in the sclerenchyma and vascular tissue of transgenic tall fescue would result in reduced leaf strength and toughness. Although feruloylation of AX may act as a nucleating site for the formation of lignin and for the linkage of lignin to the xylan/cellulose network via lignin–ferulate–xylan complexes (Jacquet et al. 1995; Bartolome et al. 1997; Iiyama et al. 1994), the decrease in esterified ferulates at the levels achieved with the transgenic plants tested here, had no direct effect on the content of lignin in transgenic, vegetative plants expressing FAEA (Buanafina et al. 2010). Control and transgenic leaves also do not differ in content of neutral or acid detergent fiber (Buanafina et al. 2010). In addition, the lignin (Klason and acetyl bromide) levels in leaves of control and transgenic lines T11 and T18, at the heading stage of development, have also been determined and did not significantly differ from control plants (data not published). Therefore, in the present study, reduced levels of ferulates cross-linked in transgenic leaves are probably responsible for their reduced strength and toughness and increased performance of FAW fed transgenic leaves. As discussed in Buanafina et al. (2010) FAEA expression *in planta* also resulted in reduced levels of *p*-coumaric acid esterified to the cell wall, in contrast with previous reports that FAEA does not act on *p*-coumaric acid. This reduced level of *p*-coumaric acid in all transgenic lines tested in the present study, did also account for the increased performance of FAW feeding on transgenic leaves. Contrary to ferulic acid, there is no evidence so far that *p*-coumaric acid units have a role to play in cell wall cross-linking. Although low levels of *p*-coumaric acid have been reported acylating AX in barley straw (Mueller-Harvey and Hartley 1986), bamboo (Ishii and Hiroi 1990) and corn bran (Allerdings et al. 2006), the high levels of *p*-coumaric acid found in grasses are known to acylate lignin not AX (Ralph et al. 1994a). Despite the fact that the majority of *p*-coumaric acid is known to acylate lignin and its accumulation seems to occur parallel to lignin deposition, lignification studies in different grass species (Hatfield et al. 2009) have shown that lignin levels are not directly related to levels of *p*-coumaric acid esterified to the cell wall. Our results are in agreement with these findings, as the reduced level of *p*-coumaric acid in transgenic lines did not translate into lower lignification. It is plausible that *p*-coumaric acid esterified to tall fescue cell walls together with ferulate monomers and dimers, limit growth of FAW by inhibiting their feeding, general metabolism and/or development, leading to reduced plant damage.

Contents in plant cells of leaves are easily digested by enzymes found in the saliva and gut of insect folivores like FAW. Although there is some minor chemical disruption of the plant cell wall, majority of the plant cell wall is indigestible by lepidopteran folivores (Martin 1991; Clissold 2008). Rate and degree of leaf fragmentation by chewing is a key factor affecting assimilation efficiency of nutrients from a plant cell and subsequent growth performance (Read

and Stokes 2006; Clissold 2008; Clissold et al. 2006, 2009). This efficiency in chewing insects like FAW is largely determined by mandible morphology and associated musculature in the head. Fractionating plant leaves with mandibles is the sole means by which caterpillars process food. Caterpillars who incise their food into smaller particles grow faster (Bernays and Janzen 1988). Our finding of higher growth rates of FAW fed transgenic tall fescue leaves compared to control leaves is likely due to higher nutrient assimilation efficiency enabled by lower food handling costs (i.e., less energy per bite) on transgenic leaves whose lower level of HCAs cross-linking AXs reduced strength and toughness. Increased access to nutrients in transgenic plants is supported by our finding that the rate of cellulase-mediated sugar release from structural carbohydrates was much higher from FAEA transgenic tall fescue leaves than from controls, suggesting that lower levels of cell wall HCA cross-links in transgenic cell walls results in more efficient release of sugars when digested with cellulase.

The association between tall fescue and fungal endophytes confers induced, indirect resistance to FAW herbivory as these endophytes produce secondary metabolites (different alkaloids) that have detrimental effect on insect invaders (Bultman and Bell 2003; Kuldau and Bacon 2008). It is likely that induced, indirect resistance had little effect in the present study because the plants used were endophyte free. Secondary metabolites produced through the phenylpropanoid pathway are also involved in indirect resistance of plants to herbivorous insects. The results from our study suggest that cell wall HCAs in small grasses are likely to function as direct defenses against herbivory acting either as antifeedants, toxic chemicals and/or as a component of the cell wall barrier (cross-linking AX and lignin) to increase toughness that deters nutrient acquisition.

The accumulation of cell wall ferulates, the cross-linking of xylan and the onset of lignification are some of the obstacles that may limit cell wall disassembly both during ruminant digestion and industrial saccharification for biofuel production. Attempts to improve cell wall degradability in order to meet industrial demands increasingly use genetic engineering to modify the grass cell wall. Transgenic lines with reduced lignin content (Wang and Ge 2006; Dien et al. 2008; Jakob et al. 2009) or reduced feruloylation (Buanafina et al. 2006, 2008, 2010) have been developed in an attempt to improve fermentability. A potential difficulty with this technological approach is that cross-linking of AX by HCAs and lignification in grass cell walls do not just function to maintain plant architecture and ensure water transport, but also seem to provide a defensive biomechanical barrier to insect herbivory (Felton 2005; Read and Stokes 2006; Clissold 2008). The positive effect demonstrated here of reduced levels of ferulate cross-linking in grass cell walls on performance of FAW larvae, a generalist insect herbivore that feeds naturally on tall fescue, confirms this biomechanical barrier as a trait that enhances resistance to herbivory by lepidopterans. Many other species of chewing insect herbivores will be exposed to these modified grasses when grown in the field because these grasses are part of the ecological food chain. Thus, releasing modified grasses with cell walls tuned for easier use in biofuel production could have unintended, positive ecological impacts on herbivores, especially grass specialists like the true armyworm [*Mythimna* (*Psuedaletia*) *unipuncta* Haworth (Lepidoptera: Noctuidae)] (Keathley and Potter 2011) that have already evolved adaptations to utilize these tough, strong grasses. Better survival of young larvae and higher nutrient assimilation leading to faster larval growth rate were observed when FAW larvae were fed transgenic grass leaves with a lower level of cell wall HCAs cross-linking AX. Population dynamics of FAW and most insect herbivores feeding on grasses is well known to be influenced by early larval survival and ability of larvae to develop rapidly to an optimal size in order to produce the largest amount of progeny possible. To avoid such increases in insect herbivory and the need of increased use of pesticides, targeted expression of FAEA and related enzymes *in planta* aimed at decreasing cell wall ferulates and diferulates in order to increase cell wall degradability, should be targeted under an inducible promoter; e.g., under a senescence promoter. This strategy would allow enzyme expression only when the plant is senescing, and consequently would not result in any change in the level of cell wall ferulates as the cell wall is being formed which is when herbivory usually occurs.

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